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Development of a Corneal Bioluminescence Mouse for Real-Time In Vivo Evaluation of Gene Therapies

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Purpose: The purpose of this study was to develop and characterize a novel bioluminescence transgenic mouse model that facilitates rapid evaluation of genetic medicine delivery methods for inherited and acquired corneal diseases.

Methods: Corneal expression of the firefly luciferase transgene (*luc2*) was achieved via insertion into the *Krt12* locus, a type I intermediate filament keratin that is exclusively expressed in the cornea, to generate the *Krt12^{luc2}* mouse. The transgene includes a multiple target cassette with human pathogenic mutations in K3 and K12.

Results: The *Krt12^{luc2}* mouse exclusively expresses *luc2* in the corneal epithelium under control of the keratin K12 promoter. The luc2 protein is enzymatically active, can be readily visualized, and exhibits a symmetrically consistent readout. Moreover, structural integrity of the corneal epithelium is preserved in mice that are heterozygous for the *luc2* transgene (*Krt12^{+/luc2}*).

Conclusions: This novel *Krt12^{luc2}* mouse model represents a potentially ideal in vivo system for evaluating the efficacies of cornea-targeting gene therapies and for establishing and/or validating new delivery modalities. Importantly, the multiple targeting cassette that is included in the *Luc2* transgene will greatly reduce mouse numbers required for in vivo therapy evaluation.

Introduction

Corneal dystrophies represent a heterogeneous group of heritable diseases in which abnormal endogenous material often accumulates in the cornea. This can result in loss of transparency, which often leads to significant vision impairment. Although many of the causative mutations have been identified, it is not possible to classify corneal dystrophies based on the mutated gene/gene product. Therefore, corneal dystrophies are classified into anatomic categories: epithelial and subepithelial, epithelial stromal, stromal, and endothelial. Corneal dystrophies are mostly inherited as autosomal dominant traits,

with few inherited as autosomal recessive or X-linked traits.¹ The autosomal dominant forms are generally caused by missense mutations or small in-frame insertion/deletion mutations in corneal epithelial cell structural molecules.^{2,3} Meesmann epithelial corneal dystrophy (MECD) is a dominant-negative corneal dystrophy caused by mutations in either of the genes encoding the corneal specific intermediate filament proteins keratin 3 (K3) or keratin 12 (K12).⁴ Mice expressing a single functional *Krt12* allele have no detectable corneal phenotype, which suggests that haploinsufficiency is not a concern for K12.⁵ Therefore, strategies aimed at transiently or permanently silencing the mutated disease-causing allele represent a viable route to therapy for MECD and other

keratinopathies.^{6,7} The well-characterized nature of MECD and the recent development of a humanized MECD phenotypic mouse model⁸ together with the fast intracellular turnover of keratin proteins,^{7,8} make this an ideal corneal disease model for the development of novel genetic therapies, including siRNA.

The cornea is an attractive tissue for gene-based therapeutics due to its accessibility and small surface area. Although many methods have been proposed to deliver genes and/or gene modifying tools to human cornea, gene therapy for corneal disorders has not yet entered the clinic. A lack of appropriate animal models for rapid assessment of therapeutic potency, specificity, longevity, and disease resolution has certainly impeded progress in this field. Tissue-specific reporter-gene animal models allow in vivo assessment of target tissue bioavailability and efficacy over time using live animal imaging.^{9,10} These models greatly reduce animal numbers required for time course experiments. We, thus, set out to develop an in vivo reporter corneal model. Here, we present the generation and characterization of a corneal bioluminescence reporter mouse that would allow researchers to assess the efficacies of several gene or mutation-specific therapeutics (e.g. siRNA, antisense oligonucleotides, etc.) in a single transgenic mouse model, offering further reductions in transgenic costs and animal use.

Materials and Methods

Multiple Target Cassette

To enable testing of mutation-specific therapeutics against several reported MECD mutations, a multiple target cassette (MTC) containing the nucleotides necessary for siRNA targeting for each of the MECD causing *KRT3* and *KRT12* mutations: K3-c.1525G>A (K3-E509K), K3-c.1508G>C (K3-R503P), K3-c.1493A>T (K3-E498V), K12-c.395T>C (K12-L132P), and K12-c.404G>C (K12-R135T) was designed as depicted in Figure 1a. Flanking 5' *NheI* and 3' *XhoI* restriction sites were added to enable directional subcloning. The MTC insert was synthesized (DNA 2.0 Inc., Menlo Park, CA, USA) and subcloned into psiTEST-LUC-target vector (York Bioscience Ltd., York, UK) to enable in vitro validation of siRNA (see Fig. 1b).

Generation of *Krt12*^{luc2} Mice

The MTC construct was inserted into the 3' UTR of the *luc2* gene in a murine genomic targeting vector designed for “knock-in” at the *Krt12* locus

(Taconic Biosciences, Köln, Germany), as shown in Figure 1c. Details of the strategy are described in the Results section. For gene targeting, the vector was linearized with *NotI* restriction digestion and transfected by electroporation into the C57BL/6N Tac ES cell line (Taconic Biosciences). Homologous recombinant ES clones were isolated and validated by PCR and Southern analysis before injection into blastocysts and re-implantation in pseudopregnant female mice. Chimeric offspring exhibiting germline transmission were bred. The puromycin resistance cassette (which could interfere with knock-in allele expression) was removed by crossing these transgenic mice to mice universally expressing *Flp* recombinase. Offspring were maintained by backcrossing on the C57BL/6 background. Mice were transferred and bred in-house with WT/heterozygote breeding pairs to achieve age-matched littermates with WT/heterozygote genotypes. Requests for this transgenic line should be addressed to corresponding author Robyn Hickerson, University of Dundee.

Mouse Genotyping

Animals were used for the following experiments in accordance with the UK Animal Welfare Act; the experiments were approved by the Home Office (Scotland). Genomic DNA (gDNA) was extracted from ear biopsies by standard protocols (65). Equimolar concentrations of a common reverse primer (K12KI.R): 5' TGA ACG GAA CTG TAC TTC TGT G 3' and either K12KI.2F: 5' ACG TCC AGA CAC AGC ATA GG 3' or K12KI.1F (5' GCT GTG GAG GCC TCT TTT C 3') were used to detect the luciferase knock-in allele (299 bp product) and/or the WT allele (553 bp product), respectively (Fig. 2a). PCR conditions consisted of an initial denaturation step for 5 minutes at 95°C, 35 cycles of 15 seconds denaturation at 95°C, 15 seconds annealing at 60°C, and 30 seconds elongation at 72°C, followed by a final elongation step of 5 minutes at 72°C using MyTaq Red DNA polymerase (Bioline, London, UK).

Live Animal Imaging

All mice used for live imaging were aged between 12 and 25 weeks. For imaging, mice were anesthetized using 1.5 to 2% isoflurane (Abbott Laboratories Ltd., Berkshire, UK) in approximately 1.5 L/min flow of oxygen. A mix of luciferin substrate (30 mg/mL D-luciferin potassium salt; Gold Biotechnology, St. Louis, MO, USA) mixed 1:1 w/v with Viscotears gel (Novartis, Camberley, UK) was dropped onto the eye of heterozygous *Krt12*^{+/luc2} transgenic mice

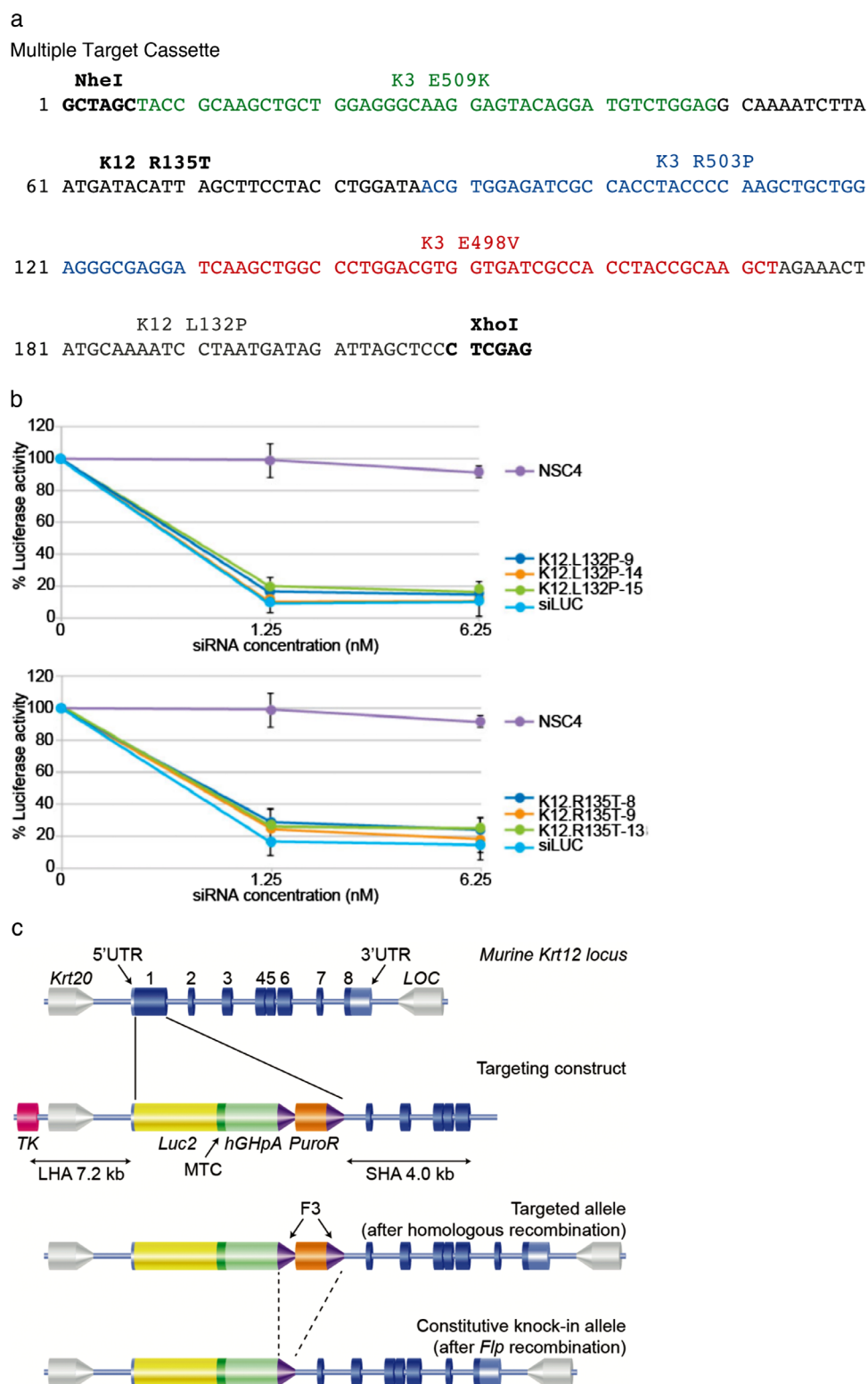


Figure 1. Generation and characterization of *Krt12^{luc2}* knock-in mouse. (a) The multiple target cassette was designed with short sequences (~40 bp each) surrounding the reported MECD causative mutations in K3 (E509K, R503P, and E498V) and K12 (R135T and L132P) were synthesized to form the multiple target cassette (MTC). (b) Mouse *Krt12* gene (exons in blue) was targeted via homologous recombination using a vector containing luciferase *luc2*. Flanking genes are *Krt20* (5') and *LOC* (3'). The targeting vector contained long and short homology arms (LHA and SHA, respectively). The construct included a multiple target cassette (MTC) within the 3' UTR, with polyadenylation

← signal *hGHpA* downstream. An F3-flanked puromycin (*PuroR*) selection cassette was included within intron 1. Transgene-positive mice were finally interbred with transgenic mice universally expressing *Flp* recombinase to remove the *PuroR* cassette. (c) Dual luciferase in vitro assays were performed to verify that mRNAs transcribed from the MTC can be targeted by mutation specific siRNAs identified for each mutation. K12 mutation-specific siRNAs were shown to inhibit reporter expression by 75 to 90% at the highest concentration evaluated. An fLUC-specific siRNA (siLUC) inhibited fLUC expression by 85-90%, whereas a nontargeting siRNA (NSC4) had no effect.

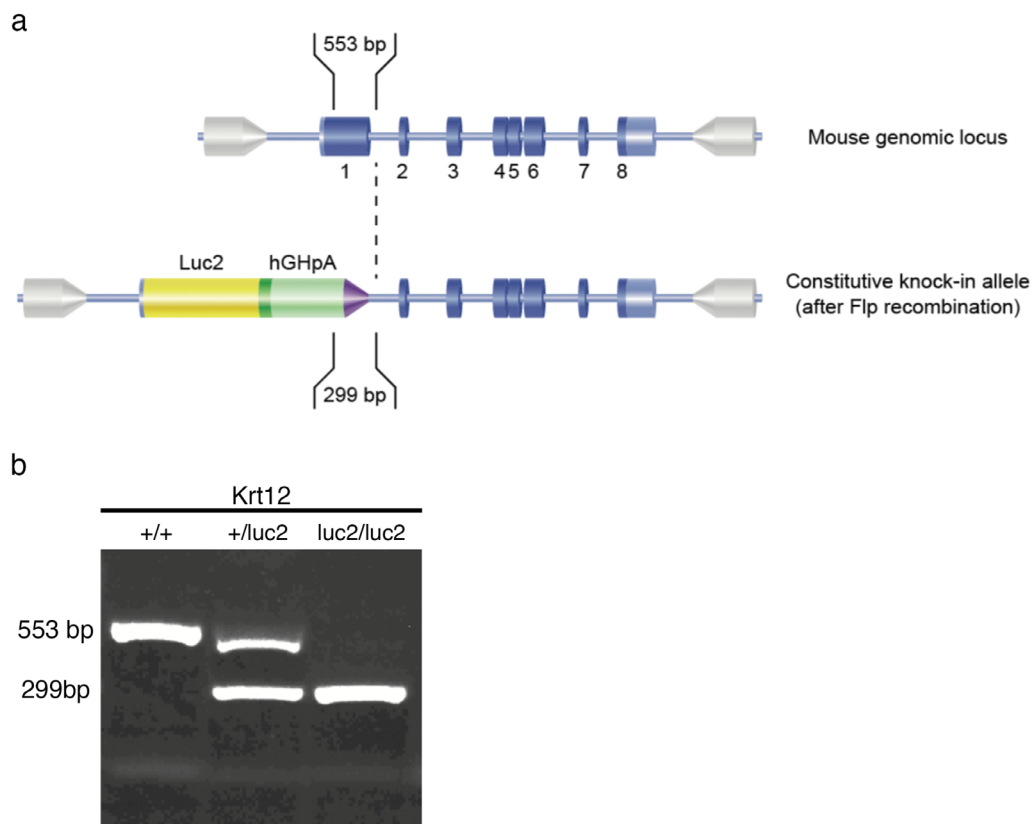


Figure 2. Mouse genotyping strategy. A common reverse primer (5' TGA ACG GAA CTG TAC TTC TGT G 3') was used with forward primer (5' ACG TCC AGA CAC AGC ATA GG 3') to detect the luciferase knock-in allele (299 bp product). Alternatively, the forward primer (5' GCT GTG GAG GCC TCT TTT C 3') detected the WT allele (553 bp product). (a) A schematic of WT *Krt12* locus and *luc2*-MTC knock-in allele shows the positions of genotyping PCR fragments. (b) Exemplar PCRs for the three possible genotypes. PCR reactions containing both bands indicated heterozygous animals.

immediately prior to imaging. A Xenogen IVIS Lumina (Perkin Elmer, Cambridge, UK) was used to quantify luminescence. A region of interest encircling the mouse eye was selected for quantification whose size and shape was kept constant throughout, using protocols as previously described.⁹

Cell Culture

Human AD293 embryonic kidney cells (Invitrogen, Paisley, UK) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal calf serum (Invitrogen). Cells were incubated at 37°C with 5% CO₂

supplement and passaged following standard laboratory procedures.

Dual Luciferase Reporter Gene Assays

For the validation of MTC functionality with native siRNA, psiTEST-LUC-MTC (fLUC reporter) was assessed with siRNAs K12.R135T-8 (5' UCUUAAUGAUACAUAUAGCU 3'), -9 (5' CUUAAUGAUACAUAUAGCUU 3'), and -13 (5' AUGAUACAUAUAGCUUCCUA 3') and K12.L132P-9 (5' AUGCAAAAUCCUAAUGAUUAU 3'), -14 (5' AAUCCUAAUGAUAGAUUAU 3'), and -15 (5' AAUCCUAAUGAUAGAUUAU 3') compared

to control siRNAs (siLUC and NSC4). All siRNAs were assessed in quadruplicate at 0, 1.25, and 6.25 nM. To confirm that human allele-specific siRNAs do not target mK12, siRNAs K12.L132P-9, and K12.R135T-5 were assessed in conjunction with control siRNAs against psiTEST-LUC-mK12 (data not shown). All siRNAs were evaluated in a range from 0 to 6.25 nM in quadruplicate.

AD293 cells were cells of choice for the luciferase assay as they do not express endogenous K12. The Dual-Luciferase Reporter Assay (Promega, Southampton, UK) was used to measure the effect of siRNA on luciferase expression according to manufacturer's instructions. The general protocol for assessment of siRNAs by dual luciferase reporter gene assay was described by Liao and colleagues.¹¹

Histopathology and Immunofluorescence

Eyes were processed for paraffin embedding, 7 μ m sections mounted on glass slides and prepared for staining as previously described.¹² Hematoxylin and eosin staining were performed following standard protocols. For detection of Luc2p, tissues were blocked with 10% (v/v) goat serum in PBS for 3 hours prior to incubation with mouse anti-Luc (L2164; Sigma USA, Chicago, IL, USA) at 1:20 dilution in blocking buffer overnight at 4°C. Sections were extensively washed between antibody incubations with PBS. Primary antibodies were detected with Alexaflour-594 conjugated anti-mouse and Alexaflour-488 conjugated anti-rabbit secondary antibodies (Molecular Probes) at 1:500 dilution in blocking buffer for 30 minutes at room temperature. All slides for immunofluorescence were mounted with Hydromount (National Diagnostics, Thermo Fisher Scientific, Waltham, MA, USA) containing 2.5% DABCO (Sigma USA) and 1 μ g/mL DAPI (Roche Diagnostics, Basel, Switzerland).

Immunoblotting

Whole-tissue protein lysates were prepared from corneal tissues snap-frozen in liquid nitrogen. Tissues were ground via mortar and pestle and reconstituted in 0.5 M Tris, 6.5% SDS, 0.1M DTT, 15% glycerol, and 0.001% bromophenol blue. Protein lysates were resolved by SDS-PAGE and processed for immunoblotting and imaged as described previously.^{12,13} Briefly, membranes were simultaneously probed with a 1:2,000 dilution of rabbit anti- β -actin (ab8227; Abcam, Cambridge, UK) and a 1:1,000 dilution of mouse anti-Luc (L2164 Sigma-Aldrich, USA). Membranes were washed, then simultaneously

probed with differentially labeled IR-dye conjugated secondary antibodies (goat-anti-rabbit 680 and goat-anti-mouse 800), washed and scanned in the 700 and 800 nm channels using an infrared imaging scanner (Odyssey Infrared Imaging System; LI-COR Biotechnology UK Ltd., Cambridge, UK).

Statistical Analysis

Statistical analysis for the in vivo siRNA experiments was performed using SPSS version 21. All error bars represent the standard error of the mean (SEM).

Results

Generation and Characterization of K12-Luciferase Multitarget Knock-in Mice

To facilitate the assessment of multiple therapeutics that target several, unique genetic mutations using a single bioluminescent mouse model, we inserted a synthetic DNA fragment containing several disease-causing mutations with their flanking sequences into the 3' UTR of the mammalian codon optimized firefly luciferase reporter gene *luc2* (see Fig. 1a). Specifically, this MTC contains a tandem array of the mutation-specific siRNA target sequences for MECD: K12 (L132P and R135T) and K3 (E509K, R503P, and E498V).

The validated *luc2*-MTC construct (see Fig. 1b) was used to generate K12 knock-in mice (Taconic Artemis, Köln, Germany) using the targeting strategy and validated using in vitro luciferase assays (see Fig. 1c). K12 is exclusively expressed in the anterior corneal epithelium,^{14,15} allowing transgene expression to be directed and restricted to this tissue. A gene-targeting vector was generated where the coding sequence of exon 1 of endogenous mouse *Krt12* was replaced with a transgene cassette containing a *luc2*-MTC gene fusion and the human growth hormone polyadenylation signal (*hGHpA*). The positive selection marker *PuroR*, flanked by F3 sites, was inserted downstream of the *hGHpA* sequence. Mouse genomic sequence from exon 2 to exon 8 of *Krt12* was left intact in order to preserve all potential regulatory elements driving expression of *Krt12*. Homologous recombinant ES cell clones (*C57BL/6NTac* ES cell line) were validated by PCR and Southern analysis and used to generate mice via blastocyst injection. Founders were crossed to mice universally expressing *flp*-recombinase to excise the *PuroR* selection cassette. Offspring that were *luc2*-positive/*PuroR*-negative were used for colony

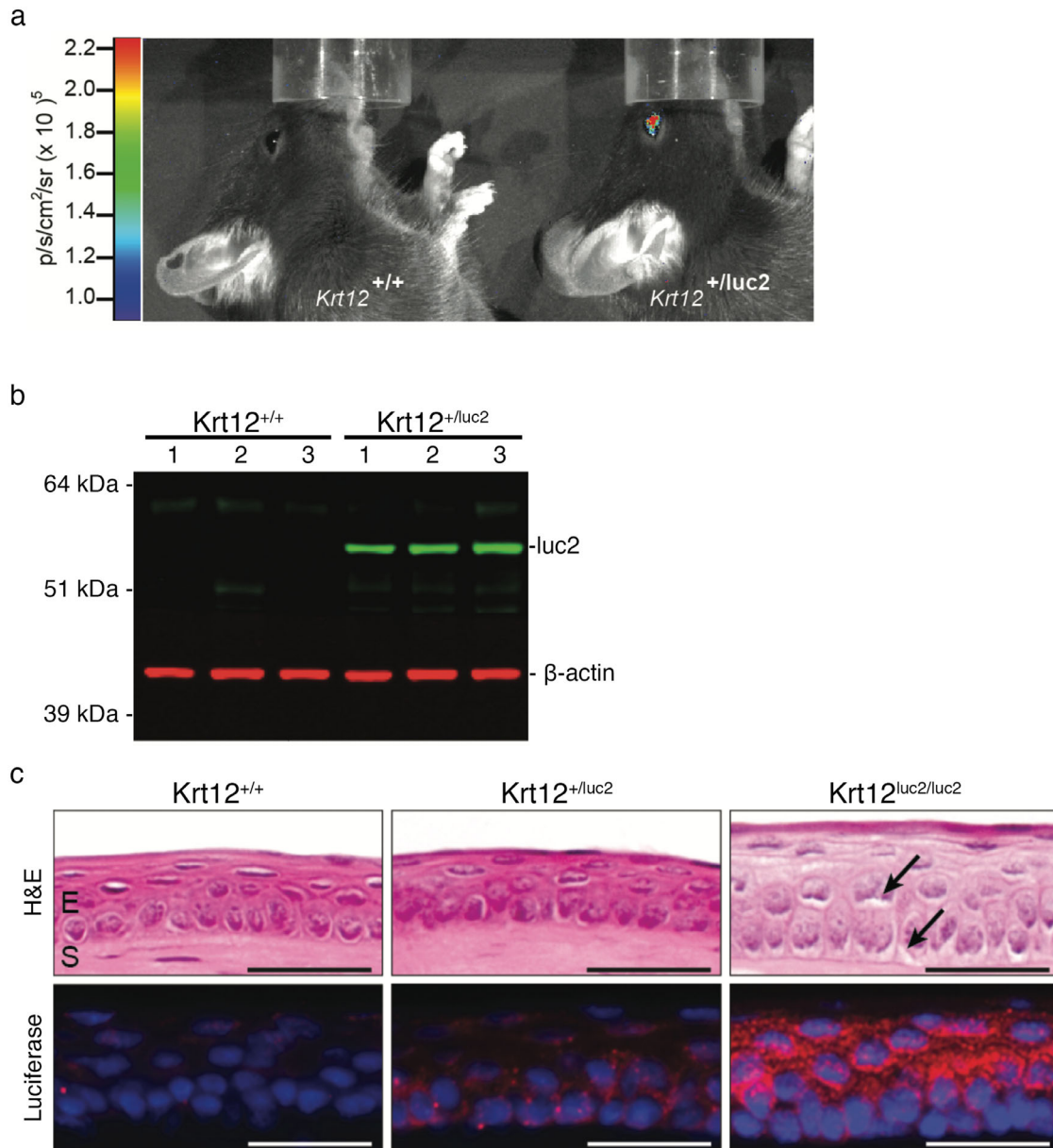


Figure 3. Characterization of *Krt12*^{luc2} knock-in mouse. (a) Bioluminescence imaging of wild-type (*Krt12*^{+/+}) and heterozygous (*Krt12*^{+/luc2}) mice with topical exposure to luciferin injected intraperitoneally with luciferin, shows bioluminescence of the eye/cornea in heterozygotes. (b) Immunoblotting of corneal protein lysates ($n = 3$ per genotype) probed with a luciferase-specific antibody (green) shows luciferase protein expression in heterozygous animals. β -actin served as a loading control (red). (c) Histology shows that WT and heterozygote corneal epithelium are indistinguishable (hematoxylin and eosin staining). However, the anterior corneal epithelium in homozygotes (which are K12- null) is thickened, with overt signs of cell fragility, notably cytoplasmic vacuoles (arrows). Immunofluorescence probing with an anti-luciferase antibody confirms corneal expression of *luc2* in both heterozygous and homozygous animals. E = epithelium, S = stroma. Scale bar = 100 μ m.

establishment. This mouse line was designated *Krt12*^{luc2} and the various genotypes are referred to as WT = *Krt12*^{+/+}, heterozygote = *Krt12*^{+/luc2}, and homozygote = *Krt12*^{luc2/luc2}. The genotyping strategy and example data are shown in Figure 2.

Luciferase transgene expression was confirmed in the cornea by real-time bioluminescent imaging of

mice that had luciferin substrate applied topically. Bioluminescent signal was detected in the eye/cornea of the heterozygous *Krt12*^{+/luc2} mice, whereas no signal was detected in wild-type (WT) animals (Fig. 3a). Immunoblotting of corneal lysates with a luciferase-specific antibody confirmed corneal expression of the reporter gene ($n = 3$ per genotype; see

Fig. 3b). Furthermore, immunofluorescence staining of paraffin-fixed sections showed luciferase protein expression in the corneal epithelia of both heterozygous and homozygous animals (see Fig. 3c). Histology showed that the corneal epithelial architecture of heterozygous animals is indistinguishable to that of WT mice; however, corneal keratinocytes from homozygotes exhibit overt morphological changes, with acanthosis and cytolysis (see Fig. 3c). This is consistent with the complete loss of K12 expression (i.e. homozygotes are equivalent to the previously reported K12-null mice), which exhibit corneal epithelial fragility.⁵

Optimization of Corneal Bioluminescence Readout

To ensure reliable therapeutic assessment, daily live animal bioluminescence measurements should be quick and consistent over time. Thus, our previously reported epidermal reporter mouse model luciferin administration was optimized for corneal bioluminescence read-outs.⁹ This optimization was required because we found that intra peritoneal (IP) luciferin injections were not a suitable route of luciferin delivery; indeed, eye drop delivery proved more suitable and provided a more stable and consistent signal.

Luciferin is highly bioavailable and is both rapidly and widely distributed. When combined with optimization of image position for the anesthetized mouse positioning, highly reproducible bioluminescence imaging of both eyes was achieved (Fig. 4a). Signal stability was further enhanced by assessing groups of animals (see Fig. 4b) and tracking the right-to-left bioluminescence ratio (R/L ratio). Figure 4c shows R/L signal stability for a group of 3 mice monitored over 5 consecutive days compared to that of the individual mice. Thus, a robust in vivo system has been defined for assessment of corneal gene silencing therapeutics using right-to-left split body study designs.

Discussion

Several hereditary diseases of the cornea are transmitted as autosomal dominant traits, and, in most cases, dominant-negative mutant proteins are expressed.^{3,11,16,17} The cornea is particularly well-suited for gene therapy due to its small size, ease of visualization, accessibility, and immune privileged status. These characteristics enable direct delivery procedures, like topical application or injection, to readily be used in the clinic.¹⁸

Recently, much effort has been expended on developing genetic therapies, including mutation-specific siRNA. The siRNA-based therapies hold great therapeutic potential for chronic inherited corneal dystrophies⁷ that could be extended to treat acute corneal conditions. Therapeutic genes have been successfully introduced into the cornea using adenoviral vectors or polyethylenimine-conjugated gold nanoparticles in animal models.^{19–22} Validation of gene delivery into the cornea in these studies was performed by qPCR or immunoblotting. Specific quantification of delivery into corneal epithelial cells and time-dependent analysis in the same animal are a major challenge with such postmortem whole tissue techniques. Immunohistochemistry on corneal explants allows assessment cellular location of transgene expression,^{23,24} and gene delivery in vivo can be assessed using a GFP reporter construct in combination with fluorescence stereomicroscopy.^{25,26} However, no single technique combines the assessment of corneal sublocalization and gene expression over time.

The scarcity of methods to specifically deliver therapeutic molecules into the appropriate cell populations of the cornea may slow progression of gene-based therapies from the laboratory to the clinic. Although gene therapy for corneal dystrophies is yet to be investigated in clinical trials (ClinicalTrials.gov, Accessed: July 2019), there are currently almost 50 interventional clinical studies underway for gene therapy of retinal diseases (ClinicalTrials.gov, Accessed: July 2019). A number of animal models are available for inherited retinal diseases, as well as in vivo imaging techniques that allow for long-term noninvasive and repeated monitoring.²⁷

To address these issues, we have developed a multi-target corneal reporter gene mouse that supports real-time in vivo assessment of therapeutic delivery into the anterior corneal epithelium. Heterozygous *Krt12*^{+/luc2} mice show no corneal pathology and the luciferase reporter gene, knocked into the *Krt12* locus, is appropriately and exclusively expressed in the corneal epithelium (see Fig. 3). The multiple target cassette within the 3' UTR of the transgene includes a range of human pathogenic mutations in K3 and K12 that we have shown to be targetable with siRNAs that inhibit mutant allele expression in vitro.^{11,28,29} This considerably expands the utility of our bioluminescent mouse model and reduces the numbers of transgenic lines required to assess personalized medicine aimed at specific mutant alleles. This methodology should find considerable utility in other genetic conditions where, like MECD, there is considerable genetic heterogeneity.

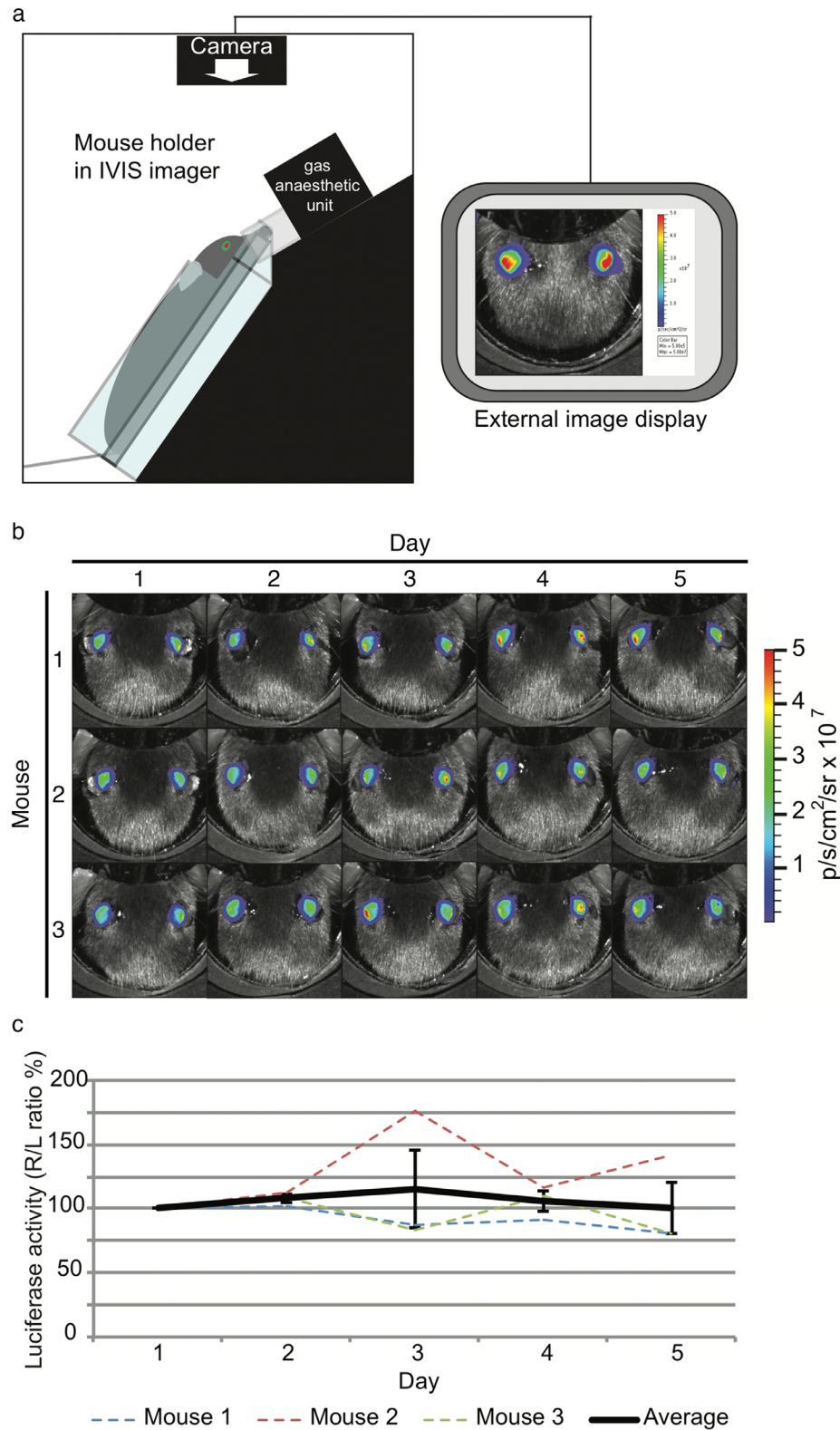


Figure 4. Optimization of bioluminescent imaging. (a) A bespoke mouse holder positioned within the Xenogen IVIS imager at an angle of 45 degrees enabled both eyes to be simultaneously evaluated in a highly reproducible manner. (b) Bioluminescence images from a group of 3 mice evaluated over 5 consecutive days. (c) Stability of bioluminescent signal from three mice. Signal from the right eye is expressed as a percentage of the left eye signal (right and left [R/L] ratio %). Signal is more stable when comparing the mean readout of all three mice (black line), as opposed to individual animals (dashed lines). Error bars represent standard error of the mean.

In summary, the multitarget bioluminescence mouse model presented here is an effective tool for real-time assessment of gene-silencing therapies and their delivery into the corneal epithelium. The *Krt12^{luc2}* mouse model represents an ideal in vivo system for iterative testing of a multitude of gene therapy and delivery system permutations, providing a robust experimental platform for translation of gene therapies into clinical use.

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* DJF and EHAA contributed equally to this work.

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